Exhibit C

U.S. Serial No. 09/308,725

to expression of the Gem protein.

The effect of deregulated expression of Gem on cell growth and morphology was investigated by permanently transfecting NIH 3T3 cells with a mycophenolic acid–selectable mammalian expression vector (pMSG) containing the entire Gem coding region (pMSG-GEM). Deregulated expression of Gem reduced the number of selectable colonies to <0.1% of that obtained with cells transfected with the vector alone (*16*). Similarly, the number of selectable colonies obtained after cotransfection of 3T3 cells with pMT2T-GEM and pSV2-neo was ~20% of that obtained after transfection with pSV2-neo alone (*2*). Transfection of pMSG-GEM into 3T3 cells previously transformed by v-*fms*, v-H-*ras*, or v-*raf* did not yield viable colonies (*16*). Because signals transduced through Raf act subsequent to those transduced through Ras (*17*), these results suggest that Gem did not inhibit growth simply by competing with Ras for substrates or regulatory factors. Rather, Gem must inhibit growth or induce cell death by some other mechanism. Gem thus appears to be a tightly regulated protein that may function in receptor-mediated signal transduction.

REFERENCES AND NOTES

1. P. Z. Zipfel, S. G. Irving, K. Kelly, U. Siebenlist, *Mol. Cell. Biol.* 9, 1041 (1989).
2. J. Maguire and K. Kelly, unpublished data.
3. C. Reynet and C. R. Kahn, *Science* 262, 1441 (1993).
4. E. F. Pai *et al.*, *EMBO J.* 9, 2351 (1990).
5. H. R. Bourne, D. A. Sanders, F. McCormick, *Nature* 349, 117 (1991).
6. M. S. Boguski and F. McCormick, *ibid.* 366, 643 (1993).
7. R. T. Miller, S. B. Masters, K. A. Sullivan, B. Beiderman, H. R. Bourne, *ibid.* 334, 712 (1988).
8. Y. W. Hwang, F. Jurnak, D. L. Miller, in *The Guanine-Nucleotide Binding Proteins: Common Structural and Functional Properties*, L. Bosch, B. Kraal, A. Parmaggiani, Eds. (Plenum, New York, 1989), pp. 77–85.
9. C. J. Der, T. Finkel, G. M. Cooper, *Cell* 44, 167 (1986).
10. B. Fletcher *et al.*, *J. Biol. Chem.* 266, 14511 (1991).
11. D. Jehner and T. Hunter, *Mol. Cell. Biol.* 11, 3682 (1991).
12. Gem mRNA abundance in primary, nontransformed cells and in tumor cell lines was determined by Northern blot analysis. Human monocytes were obtained from a normal donor by leukophoresis and purified on Ficoll-Hypaque (Pharmacia) gradients followed by countercurrent elutriation [T. L. Gerrard, C. H. Jurgensen, A. S. Fauci, *Cell. Immunol.* 82, 394 (1983)]. Monocytes were either unstimulated or were stimulated with PMA (50 ng/ml) for 22 hours before harvesting. Normal human B cells were from tonsilar mononuclear cells stimulated with *S. aureus* for 0, 2, 8, 16, 24, 48, or 96 hours; the highest Gem mRNA concentration in B cells was apparent at 2 and 8 hours. Serum-deprived human embryonic fibroblasts were stimulated with 20% fetal bovine serum as described previously (*1*).
13. The transformed cell lines that were analyzed included CEM, YT, Molt 4, Jurkat, Molt 3, H9, EL-4, ARH77, Raji, EW, BJAB, HPBALL, Nalm 6, Rch, U937, THP, HL60, K562, HEL, and HeLa. Jurkat, Molt 3, and H9 were analyzed before and after activation with PHA (1 μg/ml) and PMA (10 ng/ml). EL-4 was analyzed before and after activation with PMA (10 ng/ml).
14. P. W. Andrews *et al.*, *Lab. Invest.* 50, 147 (1984).
15. J. F. Hancock, A. I. Magee, J. E. Childs, C. J. Marshall, *Cell* 57, 1167 (1989).
16. 3T3 cells (10^5) or their transformed derivatives—the v-*fms*– and v-H-*ras*–transformed lines [M. Noda, Z. Selinger, E. M. Scolnick, R. Bassin, *Proc. Natl. Acad. Sci. U.S.A.* 80, 5602 (1983)] were obtained from R. Bassin, and the v-*raf*–transformed line was obtained from U. Rapp (both of the National Cancer Institute)—were transfected with 1 μg of either pMSG (Pharmacia) or pMSG-GEM (produced by inserting a full-length human Gem cDNA fragment into the Sma I site of pMSG) by a calcium phosphate procedure [M. Barbacid, *J. Virol.* 37, 518 (1981)]. The number of surviving colonies was determined between 10 and 21 days after selection. Approximately 100 colonies per 10^5 cells were obtained after transfection of the various cell lines with pMSG. At least 10 independent transfections (10^5 cells per transfection) were analyzed for each cell line transfected with pMSG-GEM.
17. W. Kolch, G. Heidecker, P. Lloyd, U. R. Rapp, *Nature* 349, 426 (1991).
18. T. Sekiya *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 81, 4771 (1984).
19. K. J. Horgan and S. Shaw, in *Current Protocols in Immunology*, J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, W. Strobar, Eds. (Wiley Interscience, New York, 1991), vol. 1, pp. 7.4.1.
20. J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989).
21. P. Gunning *et al.*, *Mol. Cell. Biol.* 3, 787 (1983).
22. CV-1 cells were fixed with cold methanol:acetone (50:50) on ice for 10 min and rinsed overnight in phosphate-buffered saline at 4°C. Fixed cells were labeled sequentially with mAb 2D10, fluorescein-conjugated rabbit antibodies to mouse immunoglobulin (DAKO), and fluorescein-conjugated goat antibodies to rabbit immunoglobulin (Boehringer Mannheim). NTERA-2 cells were grown on glass cover slips in Dulbecco's minimum essential medium containing 10% fetal bovine serum, and processed for fluorescence microscopy as for CV-1 cells. pMT2T-GEM was constructed by inserting the Eco RI fragment (nucleotides 1 to 1786) of clone 270-4, containing the entire open reading frame of human Gam, into the pMT2T vector (*20*). The deleted constructs were produced with nucleotide modifications to the human *gem* cDNA engineered with the polymerase chain reaction and were inserted into the pBluescript vector (Stratagene); the constructs were subsequently characterized by nucleotide sequencing of the polymerase chain reaction–generated regions and cloned into pMT2T. Transfection of COS-7 cells with pMT2T expression vectors containing either full-length or deleted *gem* cDNAs, metabolic labeling of the cells, and immunoprecipitation of cell lysates with the 2D10 mAb demonstrated the expected sizes for the engineered Gem constructs. The leader sequence for the pMT2T-delGEM[amino acids 71 to 296] was derived from T7.TagTM (Novagen). Confocal microscopy was performed with an MRC 600 Bio-Rad laser confocal scanning system and a Zeiss Axioplan microscope. Images were acquired with a 63× Zeiss planapo objective in the fast photon counting mode, enhanced with Adobe Photoshop software, and printed with a Kodak XL7700 printer.

23. We thank G. A. van Seventer for purification of CD4+ T cells, D. Webb for providing elutriated peripheral blood monocytes, J. Kehrl for a Northern blot of activated B cells, P. Scherle for a Northern blot of multiple tumor lines, and P. Davis, D. Borman, and C. Washington for technical assistance; and R. A. Kahn for discussions and advice.

7 February 1994; accepted 18 May 1994

Ability of HIV to Promote a T_H1 to T_H0 Shift and to Replicate Preferentially in T_H2 and T_H0 Cells

Enrico Maggi, Marcello Mazzetti, Adriana Ravina, Francesco Annunziato, Marco De Carli, Marie Pierre Piccinni, Roberto Manetti, Maurizio Carbonari, Anna Maria Pesce, Gianfranco Del Prete, Sergio Romagnani*

Both interferon γ (IFN-γ) produced by T helper 1 (T_H1) lymphocytes and interleukin-4 (IL-4) produced by T_H2 lymphocytes were reduced in either bulk circulating mononuclear cells or mitogen-induced $CD4^+$ T cell clones from the peripheral blood of individuals infected with human immunodeficiency virus (HIV). There was a preferential reduction in clones producing IL-4 and IL-5 in the advanced phases of infection. However, enhanced proportions of $CD4^+$ T cell clones producing both T_H1-type and T_H2-type cytokines (T_H0 clones) were generated from either skin-infiltrating T cells that had been activated in vivo or peripheral blood T cells stimulated by antigen in vitro when cells were isolated from HIV-infected individuals. All T_H2 and most T_H0 clones supported viral replication, although viral replication was not detected in any of the T_H1 clones infected in vitro with HIV. These results suggest that HIV (i) does not induce a definite T_H1 to T_H2 switch, but can favor a shift to the T_H0 phenotype in response to recall antigens, and (ii) preferentially replicates in $CD4^+$ T cells producing T_H2-type cytokines (T_H2 and T_H0).

Defects in T_H immune function can be detected in HIV-infected individuals long before the occurrence of a decline in the number of circulating $CD4^+$ T lymphocytes (*1*). Recently, it has been shown in both mice and humans that $CD4^+$ T cells represent a functionally heterogeneous population in their profile of cytokine production (*2*). T_H1 cells produce IFN-γ, IL-2, and tumor necrosis factor (TNF)–β; these cells promote macrophage activation (which re-

Division of Clinical Immunology and Allergy, University of Florence, Florence, and Division of Clinical Immunology and Allergy, University "La Sapienza," Rome, Italy.

*To whom correspondence should be addressed.

sults in delayed type hypersensitivity) and production of both complement-fixing and opsonizing antibodies. T_H2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13, provide optimal help for humoral immune responses [including immunoglobulin E (IgE)], and promote both mast cell and eosinophil differentiation and activation (2). In the absence of a prominent differentiation to T_H1 or T_H2 cells, most $CD4^+$ T cells produce both T_H1- and T_H2-type cytokines and are called T_H0 cells (3).

In previous studies, the production of IL-2, IL-4, and IL-10 has been determined in peripheral blood mononuclear cells (PBMCs) obtained from HIV-infected individuals at different stages of disease. It has been reported that enhanced production of IL-4 and IL-10 in response to stimulation with phytohemagglutinin (PHA) was associated with disease progression (4), and on the basis of these results it has been proposed that a switch from the T_H1 to the T_H2 cytokine profile plays a major role in the progression of HIV infection (4).

To examine this possibility, we used different experimental approaches (Table 1). First, we assessed the ability of PBMCs obtained from 72 HIV-seronegative healthy subjects and 73 HIV-infected individuals to produce IFN-γ and IL-4 in response to stimulation with phorbol 12-myristate 13-acetate (PMA) plus monoclonal antibody to CD3 (anti-CD3). PBMCs from HIV-infected individuals produced significantly lower amounts of both IFN-γ and IL-4 than did PBMCs from HIV-seronegative donors (Table 2). No significant differences were found in the levels of IFN-γ and IL-4 produced by PBMCs of HIV-infected individuals at different stages of infection (Table 2). Similar results were obtained by stimulation of PBMCs with PHA or a mixture of anti-CD2 and anti-CD28 (5).

One possible explanation for the discrepancy between these results and those reported by Clerici and Shearer (4) might be the different categorization of HIV-infected individuals because patients in our study with CD4 levels >500 per microliter displayed all of the different antigen-reactive groups that were seen by Clerici and Shearer (4). This possibility is unlikely because in none of our patients did PBMCs produce enhanced amounts of IL-4. Neither was the discrepancy due to lower sensitivity of the enzyme-linked immunosorbent assay (ELISA) as compared to the sensitivity of the biological assay because the ELISA can detect IL-4 concentrations as low as 5 to 10 pg/ml.

Because IFN-γ can be produced by either $CD4^+$ and $CD8^+$ T cells or natural killer cells, and in view of the fact that the levels of IL-4 detected in the supernatant of PBMCs in short-term cultures are generally low (6), cytokine secretion was also determined in T cell lines generated by stimulation with insolubilized anti-CD3 from purified $CD4^+$ T cells of 28 HIV-infected individuals and 16 HIV-seronegative healthy controls. In contrast to HIV-seronegative donors, polyclonal $CD4^+$ T cell lines from HIV-infected individuals produced significantly lower amounts of IL-4 than those produced by control T cell lines (0.23 ± 0.09 ng/ml versus 0.04 ± 0.001 ng/ml; $P < 0.05$ by χ^2 analysis). Production of IFN-γ by $CD4^+$ T cells was lower in HIV-seropositive than in HIV-seronegative subjects (4.8 ± 1.0 ng/ml versus 2.6 ± 0.5 ng/ml), but the difference was not statistically significant.

We also determined cytokine production by a panel of $CD4^+$ T cell clones generated from the peripheral blood of nine asymptomatic HIV-infected and nine HIV-seronegative individuals by a high-efficiency cloning procedure that allows the expansion of virtually every T cell (naïve, memory, resting, and activated) (7, 8). A total of 390 and 500 $CD4^+$ T cell clones were obtained from the nine HIV-seronegative donors and the nine HIV-infected individuals, respectively. All clones were assessed for IL-4, IL-5, and IFN-γ production after stimulation with PMA plus anti-CD3. The proportion of $CD4^+$ T cell clones that produced IFN-γ after stimulation was not significantly different in the two groups of subjects, whereas the proportion of $CD4^+$ T cell clones that could be induced to produce IL-4 and IL-5 was significantly reduced in HIV-infected patients in comparison with controls. This reduction was the result of the preferential depletion of both T_H0- and T_H2-type $CD4^+$ T cells in the four patients who had low numbers of circulating $CD4^+$ T cells (<200 per microliter) (Table 3).

The next approach was to assess the cytokine secretion profile of T cell clones generated from skin biopsy specimens of four HIV-infected patients. As controls, T cell clones were also generated from the skin biopsy specimens of 11 HIV-seronegative donors (four without any skin disease and seven suffering from atopic dermatitis). To selectively expand the progenies of in vivo–activated memory T cells, we cultured skin biopsy specimens in vitro with IL-2, and growing T cells were then cloned with PHA under limiting dilution conditions (9). In all, 609 clones were generated from the skin of HIV-infected patients, 330 from

Table 1. Summary of in vitro models applied to assess changes in cytokine production by cells from HIV-infected individuals in comparison with cells from HIV-seronegative healthy controls.

Type of culture	Cells involved	Changes in cytokine production	
		IFN-γ	IL-4
PBMC	T cells and non–T cells	↓*	↓
Polyclonal $CD4^+$ T cell lines	$CD4^+$ T cells	↓	↓
Cloned T cells			
PHA-induced	All T cells	■†	↓
Skin-derived, IL-2–expanded	In vivo–activated memory T cells	■	↑‡
Antigen-induced	In vitro–activated memory T cells	■	↑

*Decrease. †No difference. ‡Increase.

Table 2. Production of IL-4 and IFN-γ by fresh PBMCs from HIV-infected individuals. PBMCs (10^6 per milliliter) from 72 HIV-seronegative and 73 HIV-seropositive age- and sex-matched individuals were cultured for 3 days in RPMI 1640 medium supplemented with 5% fetal bovine serum (Hyclone Labs, Logan, Utah) in the presence of PMA (Sigma; 10 ng/ml) plus anti-CD3 (OKT3) (Ortho Pharmaceutical, Raritan, New Jersey; 100 ng/ml). Cell-free culture supernatants were collected and assayed for IFN-γ and IL-4 content by appropriate radioimmunoassay (RIA) (Cent; Centocor, Malvern, Pennsylvania) and ELISA (Quantikine R&D Systems, Minneapolis, Minnesota), respectively. Statistical analysis of the data was done by t test. HIV-infected individuals were subdivided into three groups on the basis of absolute count of $CD4^+$ T cells: >500 per milliliter, 200 to 500 per milliliter, <200 per milliliter.

Subjects	No. of cases	IFN-γ (ng/ml)	IL-4 (ng/ml)
HIV-seronegative	72	11.0 ± 1.5*	0.09 ± 0.01**
HIV-seropositive	73	6.2 ± 0.6*	0.04 ± 0.01**
($CD4^+$ T cells/ml)			
>500	24	6.2 ± 0.9	0.05 ± 0.01
<500 > 200	30	7.2 ± 1.2	0.04 ± 0.01
<200	19	4.2 ± 1.0	0.02 ± 0.01

*$P < 0.005$. **$P < 0.02$.

the skin of healthy subjects, and 443 from the skin of patients with atopic dermatitis. The majority of clones generated from both healthy subjects and patients with atopic dermatitis were $CD4^+$ (86% and 73%, respectively), the remainder being $CD8^+$ (14% and 27%, respectively), whereas only a minority (11%) of skin-derived clones in HIV-infected patients were $CD4^+$, the majority (89%) being $CD8^+$. The proportion of IFN-γ–producing $CD4^+$ T cell clones generated from the skin of HIV-infected patients was not significantly different from that of healthy controls, whereas the proportion of $CD4^+$ IFN-γ–producing clones derived from the skin of patients with atopic dermatitis was significantly lower than that of controls (Fig. 1). In contrast, the proportion of $CD4^+$ IL-4–producing clones was significantly higher both in HIV-infected patients and in HIV-seronegative patients with atopic dermatitis in comparison with HIV-seronegative healthy individuals. Unexpectedly, the proportion of $CD8^+$ IL-4–producing T cell clones generated from the skin of HIV-infected individuals was also significantly higher than that derived both from healthy subjects and from patients with atopic dermatitis (Fig. 1).

Finally, T cell clones specific for one or more *Toxoplasma gondii* (Tg) antigens were generated from the peripheral blood of three HIV-infected subjects and three HIV-seronegative healthy donors. Likewise, purified protein derivative (PPD)–specific T cell clones were generated from the peripheral blood of a fourth HIV-infected individual and from two other HIV-seronegative healthy donors. When assessed for their cytokine secretion phenotype, 40% of $CD4^+$ Tg-specific clones from HIV-seronegative subjects behaved as T_H1 cells, the remaining clones (60%) exhibiting a mixed (T_H0) profile. In contrast, virtually all Tg-specific T cell clones generated from the three HIV-infected individuals showed a T_H0 profile (Fig. 2, A and B). As expected, the majority (80%) of $CD4^+$ PPD-specific T cell clones generated from the two HIV-seronegative subjects had a clear-cut T_H1 profile, the remainder (20%) being T_H0 (Fig. 2A). In contrast, most of the PPD-specific clones derived from the HIV-seropositive donor (71%) exhibited a T_H0-like profile, and only a minority (29%) were T_H1 (Fig. 2B). No T_H2 clones specific for Tg or PPD were observed in HIV-infected donors and HIV-seronegative controls (Fig. 2A).

Taken together, these results do not support the model of a switch from the T_H1 to the T_H2 cytokine phenotype during the course of HIV infection; at most they suggest a shift in a proportion of memory $CD4^+$ T cells from the T_H1 to the T_H0 phenotype. One possible explanation for this shift is the altered cytokine production by HIV-infected antigen-presenting cells (APCs). Cytokines produced by APCs, such as IFN-α, IL-1, IL-10, and IL-12, are capable of influencing both the in vivo differentiation of naïve T cells and the in vitro development of memory T cells into a particular cytokine secretion profile (10). The production of both IL-12 and IFN-α is defective in HIV-infected subjects (*11*), despite normal or increased production of IL-1, IL-6, IL-10, TNF-α, and granulocyte-macrophage colony-stimulating factor (*12*). Therefore, the combined defect in IL-12 and IFN-α production by macrophages from HIV-infected individuals may favor the enhanced expression of T_H2 cytokines even in response to antigens, such as PPD, that preferentially expand $CD4^+$ T cell clones that have a T_H1 profile (*2*).

The results of this study also suggest a preferential depletion of $CD4^+$ T_H2-type cells in the advanced phases of HIV infection (Table 3). In favor of this finding we have observed a differential ability of T_H1 and T_H2 $CD4^+$ T cell clones to support virus replication in vitro. Fifty-two $CD4^+$ T cell clones generated from HIV-infected individuals were infected in vitro with HIV under identical experimental conditions (*13*). After 3 weeks, the presence of DNA provirus was determined by amplification with the polymerase chain reaction (PCR), and viral replication was evaluated by measurement of p24 antigen (Ag) production in the culture supernatants of the T cell clones after overnight stimulation with PMA plus anti-CD3. All of the 11 T_H2 clones and 22 out of 33 T_H0 clones produced p24 Ag.

Table 3. Cytokine production by PHA-induced $CD4^+$ T cell clones from HIV-infected individuals. $CD4^+$ T cell clones were generated from PBMCs of nine HIV-seropositive and nine HIV-seronegative subjects by stimulation of limiting numbers of T cells (0.3 per well) with PHA (0.5% v/v) in the presence of irradiated (6000 rads) allogeneic spleen cells as feeder cells (10^5 per well) and recombinant IL-2 (Eurocetus, Milano, Italy; 20 IU/ml) (*7, 8*). Clonal efficiency was calculated as described (*7, 8*). The phenotype of T cell clones was evaluated by cytofluorimetric analysis with anti-CD3, anti-CD4, and anti-CD8 (Ortho Pharmaceuticals; Raritan, New Jersey). T cell blasts from each $CD4^+$ T cell clone (10^6 per milliliter) were then stimulated for 24 hours with PMA (10 ng/ml) plus anti-CD3 (100 ng/ml) and cell-free supernatants assessed for their cytokine content. IL-4 and IFN-γ were quantitated by RIA and ELISA, respectively, as reported in Table 2. For measurement of IL-5, the murine LyH7.B13 cell line was used as indicator cells and human recombinant IL-5 (Genzyme) as a standard (*2, 9*). Cytokine levels ± standard deviations over the mean levels of controls (supernatants derived from stimulation of 10^6 irradiated feeder cells alone) were regarded as positive.

Source of T cell clones (no. of donors)	No. of clones	Clonal efficiency (%)	No. (%) of T cell clones producing:		
			IFN-γ	IL-4	IL-5
HIV-seronegative (9)					
All clones	390	55 ± 7*	284 (73)	204 (52)**	267 (68)***
HIV-seropositive (9)					
($CD4^+$ T cells/ml)					
>500 (2)	100	42 ± 5	53 (53)	50 (50)	72 (72)
>200 <500 (3)	247	44 ± 5	179 (72)	110 (44)	170 (69)
<200 (4)	153	23 ± 5*	112 (73)	36 (26)**	47 (31)***
All clones	500	34 ± 5*	344 (69)	196 (39)**	289 (58)***

*$P < 0.005$. **$P < 0.0005$. ***$P < 0.0005$.

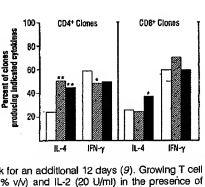

Fig. 1. IL-4 and IFN-γ production by T cell clones generated from the skin of HIV-seropositive individuals. Skin biopsy specimens obtained from four HIV-infected patients (one asymptomatic and three suffering from Kaposi sarcoma) (closed columns), four HIV-seronegative healthy volunteers (open columns), and seven HIV-seronegative subjects suffering from atopic dermatitis (shaded columns) were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 20 mM 2-mercaptoethanol, 10% fetal bovine serum (Hyclone Laboratories, Logan, Utah) (complete medium), and IL-2 (50 U/ml). IL-2 (50 U/ml) was added three times a week for an additional 12 days (*9*). Growing T cell blasts were cloned (0.3 cell per well) with PHA (1% v/v) and IL-2 (20 U/ml) in the presence of irradiated (6000 rads) feeder cells (10^5 per well). T cell blasts of each clone (10^6 per milliliter) were stimulated for 24 hours with PMA (10 ng/ml) plus anti-CD3 (100 ng/ml; Ortho Pharmaceuticals). IFN-γ and IL-4 production was assessed by RIA and ELISA, respectively, as described in Table 2. Results represent the mean percent values (± SEM) of $CD4^+$ and $CD8^+$ T cell clones producing the indicated cytokines. Statistical analysis of the data was performed with the χ^2 test (*$P < 0.05$; **$P < 0.0005$).

whereas none of the 8 T_H1 clones did. The results obtained in 6 representative clones (3 T_H1, 2 T_H0, and 1 T_H2) (Fig. 3) indicate that at least in vitro, HIV preferentially replicates in T cells that produce T_H2-type cytokines. The apparent lack of HIV replication in T_H1 cell clones did not simply reflect the ability of these clones to produce IFN-γ because the majority of T_H0 clones (which also produce IFN-γ) efficiently supported viral replication. However, IFN-γ may contribute at least in part to such a protective effect. Accordingly, in another set of experiments we found that purified $CD4^+$ T cells generated from the peripheral blood of some HIV-infected patients could be triggered to spontaneous p24 Ag production by incubation with anti–IFN-γ or anti–IFN-γ receptor (Table 4). Therefore, IFN-γ may protect $CD4^+$ T cells from HIV replication in the absence of T_H2 cytokines, possibly by acting in concert with one or more T_H1-derived soluble factors. Nonlytic suppression of HIV replication mediated by $CD8^+$ T cell–derived soluble factors has been demonstrated (*14*). Because $CD4^+$ T_H1-like cells exhibit a cytokine profile similar to that of $CD8^+$ T cells, it is possible that the same factor (or factors) are responsible for the lower efficiency of $CD4^+$ T_H1 cells in supporting HIV replication.

The preferential HIV replication in $CD4^+$ T_H2 cells may explain the reduced cloning efficiency, as well as the preferential depletion of $CD4^+$ T_H2 clones, found in the advanced phases of HIV infection (Table 3). Virus replication could indeed lead to spread of virus in culture and allow selective killing of these cells during the clonal procedure. These same mechanisms may be operating in vivo and account for a preferential depletion of T cells that produce IL-4, as suggested by the lack of IL-4 mRNA expression in freshly isolated unstimulated $CD4^+$ T cells from lymph nodes and PBMCs of HIV-infected individuals (*15*). Such a possibility is also supported by the observation that HIV-infected patients showing high IgE serum levels at the time of serodiagnosis progress more rapidly toward both the depletion of circulating $CD4^+$ T cells and the development of full-blown acquired immunodeficiency syndrome

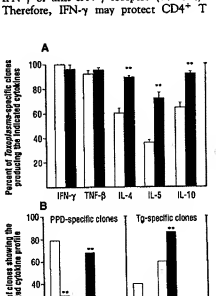

Fig. 2. Cytokine production by antigen-specific $CD4^+$ T cell clones generated from HIV-seropositive and HIV-seronegative individuals. PBMCs were stimulated for 6 days with a Tg extract (bioMérieux; Marcy-l'Etoile, France) or with PPD (Istituto Sieroterapico e Vaccinogeno Sclavo, Siena, Italy), followed by addition of IL-2 (50 U/ml). Growing T cell blasts were cloned (0.3 cell per well) in the presence of PHA (1% v/v), IL-2 (20 U/ml), and irradiated feeder cells (*2*, *9*). Tg- or PPD-specific T cell clones were identified on the basis of their ability to proliferate in response to the specific antigen under major histocompatibility complex–restricted conditions (*2*, *9*). A total of 149 $CD4^+$ Tg-specific T cell clones were obtained from the three HIV-infected patients, whereas 179 $CD4^+$ Tg-specific T cell clones were generated from the three HIV-seronegative subjects. The number of PPD-specific $CD4^+$ T cell clones generated from a fourth HIV-infected subject and from two other HIV-seronegative individuals were 34 and 79, respectively. For the induction of cytokine secretion, T cell clones were stimulated for 36 hours with PHA, and cell-free supernatants were assayed for IL-4, IL-5, and IFN-γ content as reported in Tables 2 and 3. Production of TNF-β and IL-10 was assayed by commercial ELISAs (Quantikine TNF-β; R&D, and Cytokit 10, Assay Research, College Park, Maryland, respectively). (**A**) Production of IFN-γ, TNF-β, IL-4, IL-5, and IL-10 by Tg-specific T cell clones. Results represent the mean percent values (±SE) of clones producing the indicated cytokines. (**B**) Categorization of Tg-specific or PPD-specific $CD4^+$ T cell clones according to pattern of cytokine production. T cell clones producing IFN-γ, but not IL-4, were defined as T_H1; clones producing IL-4, but not IFN-γ, were defined as T_H2; clones producing both IFN-γ and IL-4 were defined as T_H0. The mean (±SEM) percent distribution of antigen-specific T_H1, T_H0, and T_H2 clones generated from HIV-seropositive (open columns) or HIV-seropositive (closed columns) individuals is shown. Statistical analysis of the data was performed with the χ^2 test (**$P < 0.0005$).

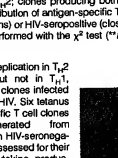

Fig. 3. HIV replication in T_H2 and T_H0, but not in T_H1, $CD4^+$ T cell clones infected in vitro with HIV. Six tetanus toxoid–specific T cell clones were generated from PBMCs of an HIV-seronegative donor, assessed for their profile of cytokine production, and infected in vitro with HIV, as reported in Table 3. Twenty days later, HIV DNA provirus was assessed by semi-quantitative PCR (*17*). Lanes a to f, calibration curve of 10^4, 10^3, 10^2, 10^1, 10^0, and 0 HIVZ6 copies added to a crude extract of 2×10^3 normal $CD4^+$ T cells. Lanes 1 to 6, 2×10^4 T cell blasts from HIV-infected $CD4^+$ T cell clones. The relative mobility of the specific product of the HIV *gag* region (115 base pairs) was defined with Hae III–digested ϕ × 174 as size marker. T cell blasts from HIV-infected clones (1×10^6 per milliliter) were then stimulated for 24 hours with PMA (10 ng/ml) plus anti-CD3 (100 ng/ml), and cell-free supernatants were assessed for p24 antigen and IL-4 content and for IFN-γ content, as reported in Table 4.

Clone number	IL-4 (ng/ml)	IFN-γ (ng/ml)	p24 antigen (pg/ml)
1	<0.05	13.2	<5
2	<0.05	16.5	<5
3	<0.05	10.5	<5
4	0.45	13.8	58
5	0.36	14.3	79
6	1.85	<0.1	82

Table 4. Induction of p24 Ag production by anti–IFN-γ or anti–IFN-γ receptor in $CD4^+$ T cells from HIV-infected individuals. T cell suspensions enriched for $CD4^+$ T cells were prepared by negative selection; PBMCs were incubated with anti-CD8, anti-CD16, and anti-CD20 followed by addition of immunomagnetic beads coated with goat anti-mouse IgG (Dynabeads M-450, Dynal, Oslo) (*13*). $CD4^+$ T cells (10^6 per milliliter) were cultured for 6 days in the absence or presence of anti–IFN-γ (1 μg/ml; IgG1: Jansen, Belgium), anti–IFN-γ receptor (1 μg/ml; IgG1: Genzyme) or control (anti-melanoma) (1 μg/ml; IgG1). As positive control, $CD4^+$ T cells were incubated with TNF-α (20 ng/ml; R&D) because of the ability of this cytokine to enhance HIV replication (*12*). Cell-free supernatants were then assayed for p24 Ag content by an appropriate ELISA (HIVAG-1 monoclonal; Abbott, Wiesbaden-Delkenheim, Germany). Data from three representative experiments are shown.

Reagent added in culture	p24 Ag production (picograms per 10^6 $CD4^+$ T cells/ml)		
	Exp. 1	Exp. 2	Exp. 3
Medium alone	<5	<5	330
TNF-α	110	164	640
Anti–IFN-γ	37	340	843
Anti–IFN-γ receptor	22	227	782
Control Ab	<5	<5	350

(AIDS) (5). We therefore suggest that progression of disease in HIV-infected individuals is not due to a switch from the T_H1 to the T_H2 phenotype, but may be favored by high and continuous HIV replication in $CD4^+$ T cells activated in vivo in response to the sustained production of T_H2-type cytokines (for example, through stimulation by common environmental allergens or helminthic infections). In contrast, some immunologic mechanism, such as activation of programmed cell death after gp120-CD4 interaction or mediated by an HIV-associated superantigen (16), may be responsible for the depletion or functional impairment (or both) of T_H1-type $CD4^+$ T cells, as observed even at the clonal level in patients with full-blown AIDS and repeated opportunistic infections (8). Thus, understanding the reasons for the selective replication of HIV may be of therapeutic value in the treatment of HIV-infected individuals.

REFERENCES AND NOTES

1. H. C. Lane *et al., N. Engl. J. Med.* 313, 79 (1985); J. L. Fahey *et al., Am. J. Med.* 76, 95 (1984); D. L. Bowen, H. C. Lane, A. S. Fauci, *Ann. Intern. Med.* 103, 704 (1985).
2. T. R. Mosman, H. Charwinski, M. W. Bond, M. A. Gledlin, R. L. Coffman, *J. Immunol.* 136, 2348 (1986); G. F. Del Prete *et al., J. Clin. Invest.* 88, 346 (1991); S. Romagnani, *Immunol. Today* 12, 256 (1991); P. Salgame *et al., Science* 254, 279 (1991); P. Parroncini *et al., Proc. Natl. Acad. Sci. U.S.A.* 88, 4538 (1991).
3. E. Maggi *et al., Eur. J. Immunol.* 18, 1045 (1988); X. R. Pallard *et al., J. Immunol.* 144, 849 (1990); R. E. Street *et al., ibid.*, p. 1629.
4. M. Clerici and G. M. Shearer, *Immunol. Today* 14, 107 (1993); M. Clerici *et al., J. Clin. Invest.* 91, 759 (1993); M. Clerici *et al., ibid.* 93, 768 (1994).
5. S. Romagnani *et al., Immunol. Rev.*, in press.
6. S. Romagnani *et al., Clin. Immunol. Immunopathol.* 50, (suppl.) 13 (1988).
7. A. Moretta, G. Pantaleo, L. Moretta, M. C. Mingari, J. C. Cerottini, *J. Exp. Med.* 158, 571 (1983); J. B. Margolick, D. L. Volkman, C. H. Lane, A. S. Fauci, *J. Clin. Invest.* 76, 709 (1985).
8. E. Maggi *et al., Eur. J. Immunol.* 17, 1685 (1987).
9. G. F. Del Prete *et al., Eur. J. Immunol.* 23, 1445 (1993).
10. C.-S. Hsieh, A. S. Heimberger, J. S. Gold, A. O'Garra, K. M. Murphy, *Proc. Natl. Acad. Sci. U.S.A.* 89, 6065 (1992); S. Romagnani, *Immunol. Today* 12, 256 (1992); R. Manetti *et al., J. Exp. Med.* 177, 1199 (1993); R. Manetti *et al., Rev. Immunol.*, in press.
11. H. E. Gendelman *et al., J. Exp. Med.* 172, 1433 (1990); J. Cherlmi *et al., ibid.*, in press.
12. G. Poli and A. S. Fauci, *AIDS Res. Hum. Retroviruses* 8, 191 (1992).
13. D. Macchia *et al., J. Immunol.* 148, 3414 (1991); D. Macchia *et al., Nature* 363, 464 (1993).
14. J. E. Brinchmann, G. Gaudernack, F. Vartdal, *J. Immunol.* 144, 2961 (1990); C. Mackewicz and J. A. Levy, *AIDS Res. Hum. Retroviruses* 9, 1039 (1992).
15. C. Graziosi *et al.*, personal communicaton.
16. J. C. Ameisen and A. Capron, *Immunol. Today* 12, 102 (1991); C. Terai, R. S. Kornbluth, C. D. Pauza, D. D. Richmen, D. A. Carson, *J. Clin. Invest.* 87, 1710 (1991); H. Giroux *et al., J. Exp. Med.* 175, 331 (1992); G. Pantaleo, C. Graziosi, A. S. Fauci, *N. Engl. J. Med.* 328, 327 (1993).
17. M. Carbonari, D. Sbarigia, M. Cibati, M. Fiorilli, *Trends Genet.* 9, 42 (1993).

18. Supported by grants from Istituto Superiore di Sanità (Progetto AIDS 1993), CNR (finalized project "Oncology"), and Associazione Italiana Ricerca sul Cancro (AIRC).

10 November 1993; accepted 21 April 1994

Lack of Evidence for the Dichotomy of T_H1 and T_H2 Predominance in HIV-Infected Individuals

Cecilia Graziosi,* Giuseppe Pantaleo, Kira R. Gantt, Jean-Pierre Fortin, James F. Demarest, Oren J. Cohen, Rafick P. Sékaly, Anthony S. Fauci

A switch from a T helper 1 (T_H1) cytokine phenotype to a T_H2 phenotype has been proposed as a critical element in the progression of human immunodeficiency virus (HIV) disease. Here, constitutive cytokine expression was analyzed in unfractionated and sorted cell populations isolated from peripheral blood and lymph nodes of HIV-infected individuals at different stages of disease. Expression of interleukin-2 (IL-2) and IL-4 was barely detectable (or undetectable) regardless of the stage of disease. $CD8^+$ cells expressed large amounts of interferon γ and IL-10, and the levels of these cytokines remained stably high throughout the course of infection. Furthermore, similar patterns of cytokine expression were observed after stimulation in vitro of purified $CD4^+$ T cell populations obtained from HIV-infected individuals at different stages of disease. These results indicate that a switch from the T_H1 to the T_H2 cytokine phenotype does not occur during the progression of HIV disease.

Two populations of $CD4^+$ T_H lymphocytes have recently been identified in mice on the basis of their mutually exclusive production of certain cytokines such as IL-2 and interferon γ (IFN-γ) (T_H1 $CD4^+$ cells) or IL-4, IL-5, and IL-10 (T_H2 $CD4^+$ cells) (*1*). A similar dichotomy between T_H1 and T_H2 cells has been identified in human $CD4^+$ T lymphocytes (*2*); however, in contrast to mice IL-10 is produced by both T_H1 and T_H2 cells in humans (*1–4*). In certain infectious diseases in mice and humans, particularly parasitic diseases, the T_H1 pattern of cytokines is associated with resistance to infection (*5*), whereas the T_H2 pattern is associated with progressive forms of infection (*5*); however, association between the T_H2 pattern and protection has been demonstrated in malaria and in certain intestinal helminth infections (5). Recently, Clerici and Shearer have investigated whether a switch from the T_H1 to the T_H2 cytokine phenotype occurs during the course of HIV infection (6). They measured the production of IL-2, IL-4, and IL-10 after in vitro stimulation with recall antigens of unfractionated peripheral blood mononuclear cells collected from HIV-infected individuals in early stages of the disease and after stimulation with phytohemagglutinin (PHA) of cells collected from patients in intermediate and late stages of the disease (6). They reported that IL-2 production decreased and IL-4 and IL-10 production increased (6) with disease progression. On the basis of these findings, they proposed that a switch from the T_H1 (IL-2 and IFN-γ) to the T_H2 (IL-4 and IL-10) cytokine phenotype is a critical step in the progression of HIV disease (6).

To address the question of a switch from the T_H1 to the T_H2 cytokine phenotype in HIV infection, we undertook several experimental approaches. (i) We performed cross-sectional analysis of the constitutive expression of a group of cytokines (IL-2, IL-4, IL-10, and IFN-γ) in unfractionated mononuclear cells isolated from peripheral blood and lymph nodes from the same HIV-infected individuals in different stages of disease. The measurement of constitutive cytokine expression ex vivo may provide important information on the predominant pattern of cytokine expression in vivo and may avoid the variability and potential for artifact that is inherent in the in vitro stimulation of heterogeneous and functionally defective mononuclear cell populations (7). (ii) We performed longitudinal analysis of constitutive cytokine expression in peripheral blood mononuclear cell samples collected from the same patient at different

C. Graziosi, G. Pantaleo, K. R. Gantt, J. F. Demarest, O. J. Cohen, A. S. Fauci, Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA.

J.-P. Fortin, Laboratory of Immunology, Institut de Recherches Cliniques de Montréal, Montréal, Québec H2W 1R7, Canada.

R. P. Sékaly, Laboratory of Immunology, Institut de Recherches Cliniques de Montréal, Montréal, Québec H2W 1R7, Département de Microbiologie et d'Immunologie, Université de Montréal, Montréal, and Department of Microbiology and Immunology, McGill University, Montréal, Québec H3T 1E2, Canada.

*To whom correspondence should be addressed.